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Advanced glycation endproducts impair endothelial progenitor cell migration and
homing via syndecan 4 shedding

Xie et al AGEs, EPC migration and syndecan 4

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Key words:

chronic inflammation; proteoglycans; advanced glycation endproducts; endothelial progenitor cells; homing

Abstract:

Endothelial progenitor cells (EPCs) are a subtype of bone marrow–derived progenitor cells. Stromal cell-derived factor 1 (SDF-1)-mediated EPC mobilization from bone marrow to areas of ischemia plays an important role in angiogenesis. Previous studies have reported that advanced glycation endproducts (AGEs), which are important mediators of diabetes-related vascular pathology, may impair EPC migration and homing, but the mechanism is unclear. Syndecan-4 (synd4) is a ubiquitous heparan sulfate proteoglycan receptor on the cell surface, involved in SDF-1-dependent cell migration. The extracellular domain of synd4 (ext-synd4) is shed in the context of acute inflammation, but the shedding of ext-synd4 in response to AGEs is undefined. Here we investigated changes in ext-synd4 on EPCs in response to AGEs, focusing on the influence of impaired synd4 signaling on EPC migration and homing. We found decreased full length and increased residue of synd4 in cells incubated with AGEs, with concomitant increase in the soluble fragment of ext-synd4 in the cell medium. EPCs from patients with type 2 diabetes expressed less ext-synd4 as assessed by western blotting. Flow cytometry analysis showed less ext-synd4 on circulating CD34⁺ PMNCs, of which EPCs form a subgroup. We then explored the role of synd4 in EPC migration and homing. Impaired migration of synd4-deficient EPCs was observed by a 2D-chemotaxis slide. Furthermore, poor homing of synd4^{-/-} EPCs was observed in a mouse model of lower limb ischemia. This study demonstrates that the shedding of synd4 from EPCs plays a key role in AGE-mediated dysfunction of EPC migration and homing.

Introduction

A growing body of evidence indicates that impaired collateralization and angiogenesis in diabetes mellitus (DM) contributes importantly to the risk of cardiovascular disease in this setting [1]. Endothelial progenitor cells (EPCs) are a subtype of bone marrow–derived progenitor cells expressing surface antigens of both hematopoietic stem cells and endothelial cells, which can mobilize from bone marrow to areas of ischemia to participate in angiogenesis [2]. In diabetic patients, impaired EPC homing is one of the major reasons why impaired formation of coronary collaterals occurs [3].

Stromal cell-derived factor-1 (SDF-1) is the most important chemokine involved in EPC homing, and exerts its effects through its classic receptor CXCR4 [4]. In response to tissue ischemia, a concentration gradient of SDF-1 is established and this induces EPC mobilization and homing to the ischemic area. Gallagher et al found that lack of SDF-1 secretion in the ischemic area contributes to EPC homing dysfunction, whilst administration of SDF-1 can promote wound healing, indicating that SDF-1-dependent signaling is critical for EPC homing [5].

Recently, it has been reported that heparin sulfate proteoglycans (HSPGs) are required for cell adhesion, migration and proliferation triggered by the combination of cytokines with their receptors [6]. Syndecan4 (synd4), a member of the HSPG family, which is synthesized in most tissues, binds to various ligands comprising extracellular matrix growth factors and cytokines [7, 8]. Several lines of evidence have shown that synd4 is involved in the SDF-1/CXCR4 axis. Hamon et al reported that synd4 can form a complex with CXCR4 on HeLa cells, human primary lymphocytes and macrophages, thereby mediating SDF-1 signaling [9]. Another study demonstrated that synd4 of the surface of HeLa cells may function as another crucial receptor of SDF-1 to promote cell recruitment in addition to its classical receptor CXCR4 [10]. Although not verified directly, it is postulated that synd4-dependent signaling may also influence SDF-1-dependent migration and homing of EPCs.

Synd4 contains an extracellular domain, a membrane-spanning region and an intracellular domain. Glycosaminoglycans are attached to the extracellular domain of

synd4, through which synd4 mediates its extracellular signaling with heparan-binding growth factors [11]. Shedding of the extracellular domain of synd4 occurs constitutively and is accelerated under certain conditions, such as acute inflammation, trauma or mechanical stress [12-14]. A variety of chemokines and cytokines in acute inflammation can induce shedding of the extracellular domain of synd4 (ext-synd4) [12, 15, 16]. Oxidized fatty acids, which are pro-atherogenic, increase the release of synd4 into the medium of cultured arterial smooth muscle cells [17]. However, little is known about the fate of ext-synd4 in response to modified biomolecules that accumulate in DM.

Advanced glycation endproducts (AGEs) are a heterogeneous group of molecules formed from the nonenzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. These accumulate in DM, and are believed to be important pathogenetic mediators of many complications of diabetes, even where glycemic control is good [18]. Our previous work and that of others suggest that AGEs impair EPC function, though the mechanism of this has not been properly elucidated [19-21]. This study was designed to explore the effect of AGEs on ext-synd4 shedding from the surface of EPCs, as well as to examine whether loss of its extracellular domain from the cell surface may lead to impairment of synd4-dependent signaling. We especially focused on the influence of impaired synd4 signaling on EPC migration and homing. Our findings suggest synd4 signaling dysfunction as a novel mechanism for impaired EPC homing in DM.

Methods:

Animals and Experimental Protocol:

All procedures with animals and cell culture were approved by the Institutional Ethics Committee of Nanjing Drum Tower Hospital (Approval No. 20011141) and performed in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (National Institutes of Health publication No. 85-23, revised 1985). C57BL/6 mice were purchased (8 weeks of age) from the Model Animal Research Center of Nanjing

University. Synd4 ^{-/-} mice were purchased from CARD Kumanoto University, and bred at the Model Animal Research Center of Nanjing University. Age-matched C57BL/6 mice served as controls. Animals were fed a standard laboratory diet with free access to food and water, and kept in a temperature- and humidity (65%–70%)-controlled room (22°C ± 1°C), with a 12:12 hour light/dark cycle. After study, all animals were euthanized by cervical dislocation under ether anesthesia.

Cell Isolation and Culture:

Human late EPCs were harvested from peripheral blood samples as described previously [20] and cultured with EGM-2 medium supplemented with growth factors and serum (EGM2 bulletkit, USA). Cells were confirmed by as late EPCs by flow cytometric analysis, Dil-acLDL uptake and tube formation assay. Detailed description of cell isolation and culture are provided in the online-only Data Supplement.

Treatment of Late EPCs with AGEs and Analysis for Synd4

Late EPCs were seeded on 6-well plates and serum starved overnight in 1% FBS. Cells were incubated with different concentrations of AGEs (Calbiochem, Germany) for two time periods (12 h and 48 h). Control cells were treated with bovine serum albumin (BSA) without glycation. Incubation concentrations and times giving the greatest effect were used in later experiments. To determine synd4 content, cells were scraped and lysed, and whole-cell lysates were treated with heparan sulfate-degrading enzymes as described [22]. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were blotted onto polyvinylidene fluoride membranes. The membranes were blocked in 5% BSA for 1 h before overnight incubation at 4°C with primary antibodies to synd4 (rabbit anti-synd4 against the cytoplasmic domain of synd4, Bioworld, USA; goat anti-synd4 against the extracellular domain of synd4, LifeSpan BioSciences, USA), as well as to β -actin (rabbit anti- β -actin, Bioworld, USA) to confirm loading of comparable amounts of protein in each lane. Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies for 1 h. The amount of synd4 was

determined using enhanced chemiluminescence and densitometry.

Late EPC treatment and western blotting

Cells were treated with N-acetylcysteine (NAC; 10mM, Sigma, USA) to inhibit oxidative stress, and receptor for AGE (RAGE) was blocked using antibody to RAGE (1:100, BD, USA). Level of reactive oxygen species (ROS) in EPCs was evaluated by DCFH-DA assay (Sigma, USA) according to the manufacturer's instructions. Cells were stimulated with SDF-1 (10ng/ml; Perprotech, USA). AMD3100 (5ug/ml; Calbiochem, Germany) was used to inhibit CXCR4. Cells were lysed, proteins extracted and separated by SDS-PAGE, and protein loading was confirmed by Coomassie blue staining. β -actin was used as loading control. Primary antibodies used were anti-synd4 (1:500, Lifespan, USA), anti-Akt (1:1000, CST, USA), anti-p-Akt (473) (1:500, CST, USA), anti-eNOS (1:1000, BD Biosciences, USA), anti p-eNOS (1:100, BD Biosciences, USA), and anti- β -actin (1:1000, Bioworld, China). Detailed descriptions are provided in the online-only Data Supplement.

CD34⁺ Peripheral Blood Mononuclear Cell (PMNC) Isolation and Flow Cytometric Analysis

The clinical study followed the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Nanjing Drum Tower Hospital (Approval No. 20011141). Written informed consent was obtained from all enrolled patients. A total of 25 patients with type 2 DM (T2DM) and 25 age- and sex-matched healthy subjects as controls were enrolled. T2DM was diagnosed as HbA1c level \geq 6.5% or fasting plasma glucose \geq 7.0 mmol/L or 2 h plasma glucose \geq 11.1mmol/L during an oral glucose tolerance test. Baseline clinical characteristics of each participant were collected, including age, sex, fasting blood glucose and HbA1c. CD34⁺ peripheral blood mononuclear cells (PMNCs) were isolated from blood using a CD34 MicroBead Kit according to the manufacturer's protocol (Miltenyi Biotec, Germany). The cells were labeled with anti-synd4 (1:25, Lifespan, USA) and Dylight 488 Goat Anti-Rabbit IgG secondary antibody (1:100, Earthox, USA). Isotype-identical

fluorescent secondary antibody served as negative controls. Cells were then analysed by flow cytometry (FACSCalibur, Becton Dickinson, USA) and Cell Quest Software counting 10,000 events per sample.

Stable Knockdown and Silencing Experiments

Lentivirus for stable shRNA integration into the host genome was purchased from Shanghai Genechem Co, Ltd. Target sequence for human Sdc4 was 5'-TGATCCTACTGCTCATGTA-3'. To achieve stable knockdown, late EPCs seeded on 6-well plates were transduced at 70%-80% confluence with lentivirus carrying a synd4 shRNA (Lv si-synd4) sequence or null (Lv null). Lentivirus also carried the puromycin-resistance and green fluorescent protein (GFP) genes. Cells were infected for 6 h in medium without serum, following which the medium was replaced with complete medium. Puromycin (1 µg/ml) was added to cells 48 hours after infection and selection occurred over 3 days. The cells were identified by CD45⁻/CD34⁺/KDR⁺. The inhibition efficiency were verified by western blot analysis (Supplemental Fig. 1). Cells were then used in the chemotaxis assay, as previously reported [23]. Detailed descriptions are provided in the online-only Data Supplement.

Cell Preparation and Transplantation

Mouse EPCs were harvested from femurs and tibias of C57BL/6 and synd4^{-/-} mice as described previously [5], and cultured with EGM-2 medium (EGM2; Lonza, USA) supplemented with murine vascular endothelial growth factor (10 µg/L; VEGF; Peprotech) and 10% fetal bovine serum (FBS; Gibco, USA). Cell identity was confirmed by flow cytometric analysis and Dil-acLDL uptake (Supplemental Fig. 2), and cells were labeled with the lipophilic fluorochrome chloromethyl benzamidodialkyl carbocyanine (CM-Dil, Molecular Probes) according to the manufacturer's instructions. A mouse model of critical limb ischemia was established as described previously [19], and mice with limb ischemia were injected with 5x10⁶ EPCs via neighboring normal tissue. Two weeks after EPC transplantation, animals were euthanized and histological analysis was undertaken to assess EPC homing and

angiogenesis. Detailed descriptions are provided in the online-only Data Supplement.

Statistics

SPSS 16.0 was used for all statistical analyses. Data are presented as mean \pm SEM of three independent experiments unless otherwise stated. The significance of changes was evaluated using analysis of variance and Tukey's post-hoc test. Values of $P < 0.05$ were considered statistically significant.

Results:

Characterization of Late EPCs

Late EPCs isolated from human blood samples were proliferated in culture. Dil-acLDL uptake assay was used to further confirm the EPC phenotype. After co-culture with Dil-acLDL, the cells were positive for Dil-acLDL and concomitant BS-1 lectin (Fig.1A). Flow cytometric analysis showed these cells to be positive for KDR (84.4%) and CD34 (51.2%), and negative for CD45 (0.11%) (Fig.1B). Then, these cells showed tube formation on matrigel (Fig.1C). Taken together, these data show the cells in these culture conditions to be predominantly late-stage EPCs.

Shedding of Ext-Synd4 is accelerated in late EPCs following incubation with AGEs.

We determined whether late EPC incubation with AGEs accelerated shedding, and decreased cell surface protein level of ext-synd4. EPCs were cultured and incubated with different concentrations of AGEs (50, 100, 200, 400 μ g/ml) for 12 h. Synd4 was detected by western blotting using an antibody against an extracellular epitope. Results showed that the expression of ext-synd4 on cells decreased as the concentration of AGEs increased, with the most effective concentration being 200 μ g/ml (Fig.2B). Furthermore, when the stimulation time was extended to 48h, similar result about ext-synd4 decrease on cells was observed (Fig.2C). Next, we used ELISA to measure soluble synd4, which was shedded from cells in the conditioned medium. We found that, after 12 h stimulation, soluble synd4 in the medium of treated

cells increased by 30% compared with control (Fig.2D). These results indicated that synd4 was shedded from HUVECs, contributing to increased protein level in the medium and decreased on cell surface.

Furthermore, we detected synd4 by western blot using an antibody against a cytoplasmic epitope. As previously reported [22], the synd4 fragment of about 10 kDa represented the residual fragment that constituted the cellular part of synd4 after shedding. Meanwhile, the fragment of about 40 kDa represented full length protein. Full length synd4 (about 40 kDa) decreased after AGE treatment. Concomitantly, the fragment about 10kDa was found to be increased after AGEs administration (Fig.2E). These data demonstrate that shedding of ext-synd4 is accelerated in response to AGEs, giving rise to decreased expression of ext-synd4 on the cell surface.

RAGE and ROS are Involved in AGE-Dependent Ext-synd4 Shedding.

As shown in Fig 2B and C, ext-synd4 shedding induced by AGEs occurred in a dose-dependent manner, with a maximum effect seen at 200 µg/ml concentration, consistent with a receptor-mediated effect. The multi-ligand receptor for advanced glycation end products (RAGE) was the first, and has been the most studied, receptor for AGEs. To investigate whether the mechanism of synd4 shedding involved RAGE, it was blocked using a blocking antibody (anti-RAGE) and ext-synd4 was measured by western blotting. We found that ext-synd4 shedding induced by AGEs was largely but not completely inhibited by anti-RAGE (Fig.2F). The metabolic derangements in DM enhance production of ROS and trigger pro-inflammatory signaling. We postulated that ext-synd4 shedding would likely be regulated by ROS level. We used NAC to inhibit oxidative stress in cells, and found that shedding of ext-synd4 induced by AGEs could be rescued in the presence of NAC. ROS levels in cells were tested using DCFH-DA. We found that AGEs significantly increased ROS by late EPCs, whilst in the presence of NAC or of anti-RAGE ROS generation was decreased (Fig.2G). The shedding of ext-synd4 was positively associated with ROS level. These data suggest that the shedding of ext-synd4 may be accelerated through AGE/RAGE/ROS signaling.

Ext-synd4 on the Surface of Late EPCs and PMNCs is Decreased in T2DM Patients

As above, we found decreased ext-synd4 on the surface of late EPCs following incubation with AGEs in vitro. We went on to explore the level of ext-synd4 on the surface of late EPCs in patients with T2DM (Supplemental Tab 1). Late EPCs were isolated from T2DM patients. Following lysis of the cells, we detected and measured ext-synd4 by western blotting. We found ext-synd4 to be significantly decreased in late EPCs from T2DM patients compared with those from control subjects (Fig.3A).

We also wished to specifically determine surface expression of ext-synd4 on circulating late EPCs. In view of the sparsity of CD34⁺/KDR⁺ cells in peripheral blood, we analyzed circulating CD34⁺ PMNCs, of which EPCs form a subgroup, by flow cytometry. We found that surface ext-synd4 on circulating CD34⁺ PMNCs was decreased in T2DM (Fig.3B).

Taken together, these data demonstrate that AGEs induce reduction of ext-synd4 expression on the surface of EPCs in T2DM owing to increased shedding.

Synd4 Regulates Late EPC migration.

To verify the function of synd4 in late EPC migration, synd4 was specifically down-regulated using RNAi. Late EPCs were transduced with Lv si-synd4 or Lv null. Specific RNAi decreased synd4 protein synthesis by ~60%. Lv null-transduced late EPCs were also incubated with AGEs or control. Chemotaxis assay revealed that cells of all groups moved along an SDF-1 (0-5ng/ml) gradient. Forty cells of each group were selected randomly and the movement of the cells was tracked (Fig.4A). All the cells in the three groups were moved in a chemotactic gradient. The p-value of the Rayleigh test in logarithmic scale was below 0.05. Synd4 knockdown in late EPCs significantly reduced the cell migration, similar to the movement of cells incubated with AGEs. The euclidean and cumulative distances were decreased in synd4 deficiency and AGEs treated EPCs, compared with WT EPCs, suggesting defective motility, (Fig.4A, B and C, and Supplemental Videos).

It has previously been demonstrated that SDF-1 is the critical chemokine for EPC homing [24]. We explored the role of synd4 in SDF-1-dependent EPC homing. The siRNA was used to decrease synd4 expression (Fig.4F). CXCR4 and CXCR7 were two receptors of SDF-1 on the EPCs surface. SDF-1 could induce CXCR4 internalization and CXCR7 externalization. Synd4 knockdown and lenti-virus treatment had no effect on CXCR4 and CXCR7 alternation in the late EPCs (Fig. 4D and E). We found that SDF-1 stimulation of late EPCs induced both Akt and eNOS phosphorylation (an index of their activation). Synd4 down-regulation could blunt SDF-1-induced phosphorylation of both Akt and eNOS (Fig.4G and H). Since CXCR4 has been reported as the classical receptor for SDF-1, we used AMD3100 to inhibit CXCR4, and found that SDF-1-induced Akt and eNOS phosphorylation were also decreased (Fig.4G and H). The reduction of Akt and eNOS phosphorylation was not different in cells with synd4 knockdown compared with cells treated with AMD3100. However, when synd4 and CXCR4 were abrogated simultaneously, both Akt and eNOS phosphorylation were further reduced compared with cells with synd4 deficiency or AMD3100 treatment alone. These data indicate that synd4 may act as an independent receptor to regulate SDF-1-dependent EPC migration.

Synd4 is Necessary for Late EPC Homing

Since we found synd4 could mediate EPC migration in cultured cells, we next established a lower limb ischemic model, to investigate whether lack of synd4 would impair EPC homing. BM-derived EPCs was isolated from synd4-deficient mice. EPCs from corresponding wild-type (WT) mice were used as control. EPCs without synd4 revealed similar CXCR4 expression, poor migration and decreased eNOS phosphorylation compared with WT EPCs (Supplemental Fig. 3). EPCs tagged with CM-Dil were injected into the ischemic lower limb through neighboring normal tissue. Fluorescence analysis showed that the number of EPCs homing to the site of injury in synd4-deficient mice was considerably lower than that in controls (Fig.5A). To clarify whether EPC homing had an effect on angiogenesis in ischemic tissue, we labeled vessels with CD31 and vWF. Fluorescence analysis indicated that the vascular

density in mice injected with WT EPCs was higher than in mice injected with synd4-deficient EPCs (Fig.5B and C). Meanwhile, we assessed immune cell in the ischemic tissue by immunofluorescence with CD45. No difference of CD45 positive cell was observed among three groups (Supplemental Fig. 4). These data demonstrate that synd4 plays an essential role in EPC homing for wound healing and vessel formation in vivo.

Discussion

The current study was designed to dissect the role of synd4 in EPC migration and homing in the presence of AGEs. The four main findings were as follows: (1) The shedding of ext-synd4 on EPCs was accelerated by AGEs. (2) Ext-synd4 was decreased on the surface of EPCs following prolonged exposure to AGEs. (3) Synd4 deficiency impaired SDF-1-dependent EPC migration. (4) Synd4 deficiency impaired EPC homing. It was likely that deficiency of ext-synd4 on the surface of EPCs as a result of sustained shedding in response to AGEs may be partly responsible for the impairments in both EPC homing and angiogenesis in DM.

Various agents can induce synd4 shedding in the context of, for example, acute injury, wound healing and atherosclerosis [12, 14, 16, 17, 22, 25]. Normally, syndecan shedding occurs through direct proteolysis (e.g. thrombin) or receptor-mediated activation of sheddases (e.g. epithelial growth factors). It is still not fully clear how extracellular stimuli influence sheddases to mediate syndecan cleavage. Different stimuli appear to activate distinct intracellular signaling pathways, and increased oxidative stress is thought to play an important role in sheddase activation and syndecan shedding [26, 27]. AGEs are a complex and heterogeneous group of abnormal metabolic compounds that accumulate in DM. Here, we demonstrated for the first time that AGEs could induce synd4 shedding, and reported that increase in ROS, induced by AGEs, played a part in the process of shedding.

We found that soluble ext-synd4 in the conditioned medium increased, and cell surface ext-synd4 reduced, 12 h after incubation with AGEs. Western blotting using an antibody recognizing a cytoplasmic epitope of synd4 (cyt-synd4) demonstrated a

10 kDa fragment, representing the residual cellular part of synd4 after shedding, in cells treated with AGEs. When AGE incubation was prolonged to 48h, the expression of ext-synd4 was still decreased. Similar results regarding synd4 turnover have been reported by Strand et al [22]. In their study, pro-inflammatory cytokines, including TNF- α , IL-1 β and lipopolysaccharide, were used to treat myocytes and fibroblasts; western blotting showed the presence of a 10kDa fragment as detected by antibody against cyt-synd4, while nothing was detected using antibody against ext-synd4, suggesting that ext-synd4 was decreased on the cell surface due to shedding. Ext-synd4 expression depends on the balance between synd4 synthesis and shedding. Ramnath et al. found that synd4 mRNA of ECs significantly increased 1 h after stimulation with TNF- α and decreased to normal after 24 h [28]. Extradomain shedding paralleled synd4 synthesis initially, but was more sustained and greater in magnitude later on, suggesting that the compensatory synthesis of synd4 may not catch up with the speed of consumption, giving rise to ext-synd4 deficiency following persistent stimulation. In this study, residual cellular part of synd4 was increased after AGEs treatment, indicating increased synd4 gene expression. Meanwhile full length and extracellular epitope of synd4 were decreased, indicating more sustained and greater extracellular epitope shedding induced by AGEs.

Besides synd4, syndecan 1 (synd1), another member of the syndecan family, was reported to be decreased on the cell surface in response to inflammation. In a pig ischemia/reperfusion model, the extrdomain of synd1 (ext-synd1) and other heparan sulfates were shedded, resulting in aberrant endothelial heparan sulfate expression [29]. In another study, the content of ext-synd1 was found to be decreased in the vasculature of doxorubicin-treated hearts [30]. When lipopolysaccharide was used to induce sepsis in rats, decreased ext-synd1 together with up-regulated matrix metalloproteinases 2 and 9 were observed in aortic homogenates [31]. We therefore propose that synd4 and other syndecan family members are highly sensitive to proteolytic degradation and shedding under inflammatory conditions, and when such conditions persist, the extrdomain of these proteins decrease on the cell surface, resulting in altered cell phenotype.

Several studies have reported that ext-synd4 can bind SDF-1 directly to mediate SDF-1-dependent cell migration [9, 10]. Whether ext-synd4 shedding contributes to impaired EPC migration in response to SDF-1 has not been known. Here we observed that cell migration in an SDF-1 gradient was impaired in parallel with loss of ext-synd4 from the surface of EPCs in response to AGEs, and this phenotype was reproduced in cells by genetic ablation of synd4. We also found that SDF-1-dependent signaling pathways were impaired in synd4-deficient cells. These results indicated that loss of ext-synd4 alters synd4-dependent cell migration.

SDF-1 plays an important role in the mobilization of cells from the bone marrow and may also regulate the mobilization and recruitment of EPCs [4]. The biological effect of SDF-1 was reported to depend mainly on CXCR4, a specific G protein-coupled receptor [32]. Synd4 was shown to bind directly with SDF-1, and form a heteromeric complex with CXCR4 at the plasma membrane of cells [9]. Glycosaminoglycans (GAGs) bind SDF-1 and facilitated the binding of SDF-1 to CXCR4. When GAGs were blocked by β -D-xyloside, cell chemotaxis induced by SDF-1 was found to be decreased significantly [33]. Another study reported that the cytoplasmic tail of synd4 played an important role in SDF-1-mediated signal transduction. SDF-1 can induce synd4 tyrosine phosphorylation, which was co-associated with tyrosine phosphorylated CXCR4 [10]. In our study, we observed SDF-1 downstream signaling activation was suppressed by either down-regulation of synd4 or inhibition of CXCR4. Combined synd4 silencing and CXCR4 inhibition was found to have a synergistic effect on SDF-1 signaling. Our data suggested that synd4 behaves as an SDF-1 receptor to mediate EPC migration.

Conclusion:

This study provides evidence for the first time that AGEs induce accelerated shedding of ext-synd4, which in turn leads to impaired EPC migration and homing. Our findings suggest that decreased ext-synd4 on the EPC surface is a novel mechanism by which AGEs induce EPC homing dysfunction.

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Disclosures

The authors declared that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Figure legend:

Figure 1. Late EPCs are identified by flow cytometry and Dil-acLDL uptake assay. (A) Dil-acLDL uptake assay shows the cells were positive for Dil-acLDL and concomitant BS-1 lectin. N=3. Bar: 25um. (B) Flow cytometric analysis reveals the cells, isolated from human blood sample, are positive for KDR and CD34, and negative for CD45. (C) Representative image showing tube formation of late EPC in matrigel. N=3. Bar: 100um.

Figure 2. AGEs induce synd4 shedding from late EPCs. (A) Schematic showing the two antibodies against the extracellular domain and cytoplasmic epitope of synd4. (B and C) Representative immunoblots and quantitative data showing ext-synd4 level in late EPCs following 12 h (B) or 48 h (C) incubation time with different concentrations of AGEs using antibody against ext-synd4. (D) Soluble ext-synd4 levels in the cell culture medium were measured by ELISA. (E) Representative immunoblots and quantitative data showing cyt-synd4 protein expression in late EPCs incubated with 200 µg/l AGEs for 12 h using antibody against cyt-synd4. The fragment of about 10 kDa was increased, representing the residual fragment of synd4 after shedding; in parallel, the full length fragment (40 kDa) of synd4 was decreased. (F) Representative immunoblots and quantitative data showing that the decrease in ext-synd4 in late EPCs following AGEs incubation was attenuated in the presence of anti-RAGE antibody or N-acetylcysteine (NAC). (G) Fluorescence intensity and quantitative data showing that the ROS level in late EPCs was increased after AGEs incubation, and decreased when anti-RAGE antibody or NAC was co-incubated. Original magnification, ×200. N=3. *, $p<0.05$, compared with the group incubated with BSA; #, $p<0.05$, compared with the group incubated with AGEs.

Figure 3. Ext-synd4 is diminished on late EPCs and CD34⁺ PMNCs from patients with T2DM. (A) Representative immunoblots and quantitative data showing that ext-synd4 expression was decreased in late EPCs from T2DM patients compared with those from controls. N=6. (B) Representative flow cytometric and quantitative data showing

that surface ext-synd4 expression was decreased in CD34⁺ PMNC from T2DM patients compared with those from controls. N=6, *, p<0.05, compared with the control group.

Figure 4. Inhibition of synd4 reduces the migration of late EPCs. Late EPCs were treated with Lv null, Lv si-synd4, or Lv null together with AGEs, and their migration was examined. (A) Movements of the three groups of late EPCs in an SDF-1 gradient (0-5 ng/ml) were observed simultaneously by fluorescence microscopy. Forty cells of each group were selected randomly, tracked and analyzed. The quantitative evaluation of cumulative (B) and Euclidean (C) distances is displayed in the lower panels. (D and E) Representative immuno-fluorescence and quantitative data revealed that SDF-1 induced CXCR4 internalization and CXCR7 externalization. No alternation of CXCR4 and CXCR7 was observed when synd4 was down-regulated. (F to H) Representative immunoblots and quantitative data revealing the downstream pathways induced by SDF-1 in late EPCs. Synd4 expression was suppressed by Lv si-synd4 treatment (F). SDF-1 induced phosphorylation of Akt and eNOS, which was blunted by synd4 deficiency and/or incubation with AMD3100 (G and H). *, p<0.05, compared with the group of Lv null. #, p<0.05, compared with the group of SDF-1. &, p<0.05, compared with the corresponding control. Bar=50um, MFI: mean fluorescence intensity.

Figure 5. Synd4 deficiency blunts EPCs homing. (A) Representative fluorescence microscopy and quantitative data showing impaired EPC homing into ischemic lower limb through neighboring normal tissue. (B) Representative fluorescence microscopy and quantitative data revealed that CD31 positive vascular density was decreased in animals injected with synd4-deficient EPCs compared to those injected with wild-type (WT) EPCs. (C) Representative fluorescence microscopy and quantitative data showing that vWF positive vascular density was decreased in animals injected with synd4-deficient EPCs compared to those with WT EPCs. N=6, Bar=100um in A, 50um in B and 200um in C, *, p<0.05, compared with the corresponding control.

Graphical Abstract:

AGEs induce syndecan 4 (synd4) shedding from late EPCs. The synd4 was an important receptor on the cell surface to mediate cell migration. (A) Schematic showing the antibody against the cytoplasmic epitope of synd4. (B) Representative immunoblots showing synd4 expression in late EPCs incubated with 200 µg/l AGEs for 12 h using antibody against cyt-synd4. The fragment of about 10 kDa was increased, representing the residual fragment of synd4 after shedding; in parallel, the full length fragment (40 kDa) of synd4 was decreased. N=3. (C) Representative flow cytometric showing that surface ext-synd4 expression was decreased in CD34⁺ PMNC from T2DM patients compared with those from controls. N=6. (D) Late EPCs were treated with Lv null, Lv si-synd4, or Lv null together with AGEs, and their migration was examined in an SDF-1 gradient (0-5 ng/ml) by fluorescence microscopy. Forty cells of each group were selected randomly, tracked and analyzed. Inhibition of synd4 reduces the migration of late EPCs. N=3.